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(54) Title: SUSTAINED AND CONTINUOUS PRODUCTION OF HIGH TITERS OF RECOMBINANT VIRAL VECTORS AND TRANSDUCED TARGET CELLS FOR USE IN GENE THERAPY

(57) Abstract

This invention provides improved methods for producing high titers of recombinant viral vectors by culturing producer cells in a hollow fiber bioreactor. The titer of virus in the extra fiber space (EFS) of the hollow fiber bioreactor is sufficient to transduce target cells at a multiplicity of infection sufficient to render the transduced target cells useful for genetic therapy. In a preferred embodiment, target cells are transduced with the EFS medium from a hollow fiber bioreactor in which a producer cell line, which releases packaged retroviral vectors into the EFS, has been cultured. Lymphocytes derived from an adenosine deaminase (ADA)-deficient individual that have been transduced with EFS medium that contains retroviral vectors that include RNA that encodes ADA, express ADA at a rate comparable to that of cells from an individual who does not have ADA deficiency.

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SUSTAINED AND CONTINUOUS PRODUCTION OF HIGH TITERS OF RECOMBINANT VIRAL VECTORS AND TRANSDUCED TARGET CELLS FOR USE IN GENE THERAPY

FIELD OF INVENTION

This invention is directed to methods for producing high titers of viral vectors in vitro in a hollow fiber bioreactor, to methods for infecting target cells at high multiplicity and for producing high concentrations of transduced target cells, and to the transduced target cells produced by the methods. The methods are particularly suited for producing transduced target cells for use in methods of genetic therapy.

BACKGROUND OF THE INVENTION

Genetic therapy.

Genetic therapy for treatment of acquired and inherited diseases is a recent and highly promising addition to the repertoire of treatments for such diseases. It is expected that many congenital genetic abnormalities and acquired diseases will be amenable to treatment by genetic therapy. Genetic therapy can be effected by removing selected cells, target cells, from an afflicted individual, modifying the cells by introducing heterologous DNA that encodes a therapeutically effective product and returning the modified cells to the individual. Eventually it may be possible to introduce the heterologous DNA directly into cells in vivo, such as endothelial cells that line the lungs, without any in vitro manipulation of the target cells.

Diseases that are candidates for such treatment include those that are caused by a missing or defective gene that normally encodes an enzyme, hormone, or other protein. Examples of such diseases include: a severe combined immunodeficiency disorder, which is caused by a

defect in the DNA that encodes adenosine deaminase (ADA) (see, e.g., Kredich et al. (1983), p. 1157, in The Metabolic Basis of Inherited Disease (5th ed.), eds. Stanbury, et al., McGraw-Hill, New York); Lesch Nyhan disease, which is caused by a defect in the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT); cystic fibrosis and Duchenne muscular dystrophy for which the respective defective genes have recently been identified; Tay sachs disease; and hemoglobin disorders, such as β-thalassemia. In addition, genetic therapy has been proposed as a means to deliver therapeutic products, such as tumor necrosis factor (TNF) for the treatment cancers and CD4 receptor protein for the treatment of AIDS (see, e.g., PCT International Application No. WO 90/01870).

Genetic therapy involves introducing heterologous DNA into at least some cells of a host organism in a manner such that the products encoded by the heterologous DNA are expressed in the host. Upon introduction into the host cell, the heterologous DNA may be integrated into the genome of the host cells or it may be maintained and replicated as part of an episomal element. The heterologous DNA may encode products that replace or supplement the product of a defective or absent gene or a gene that is normally expressed at low levels or the DNA may encode therapeutic products that are effective for treating a disease. The heterologous DNA is operatively linked to a promoter and/or other transcriptional and translational regulatory elements that are recognized by host cell effector molecules, such as RNA polymerase II, such that it can be expressed in the host cell. As understanding of the underlying genetic bases for disease increases, it will be possible

to refine the methods of genetic therapy so that regulatory controls that operate at the level of gene transcription or translation or that rely on mechanisms, such as feedback inhibition, to control expression of gene products can also be provided to the host cells. For example, the heterologous DNA may also mediate or encode RNA or protein products that mediate expression of a host cell gene or biochemical process. Expression of the heterologous DNA can thereby be fine-tuned to the needs of the afflicted host.

It is also anticipated that numerous means for introducing heterologous DNA into the cells and genomes of individuals will be developed and refined. At the present time, the use of recombinant viral vectors, which are derived from viruses that infect eukaryotic cells, provide the most promising means for effecting genetic therapy. Generally, upon infection of a eukaryotic host, a virus commandeers the transcriptional and translational machinery of the host cell. In order to do so, viral regulatory signals, such as promoters, particularly those recognized early in infection, tend to be highly efficient so that any DNA that is in operative linkage with such promoters and regulatory signals is efficiently expressed at high levels. Eukaryotic viruses have, therefore, been used as vectors for cloning and expression of heterologous DNA in eukaryotic cells.

Recombinant eukaryotic viruses for delivery of heterologous DNA.

Eukaryotic viruses from which recombinant viral vectors have been constructed include both DNA viruses, such as SV40, adenovirus, and bovine papilloma virus (see, e.g., Gluzman, Y., ed. Eukaryotic Viral V ctors,Cold Spring Harbor Laboratory, Cold Spring

Harbor, NY (1982); Sarver et al. (1981) Mol. Cell Biol. 1: 486; and U.S. Patent No. 4,419,446 to Howley), and RNA viruses, retroviruses, such as Moloney murine leukemia virus (MoMLV), mouse mammary tumor virus (MMTV), Rous sarcoma virus (RSV) and other leukemia and tumor viruses (see, e.g., Mann et al. (1983) Cell 33: 153-159; Miller et al. (1986) Mol. Cell. Biol. 6: 2895-2902; U.S. Patent No. 4,868,116 to Morgan et al.; U.S. Patent No. 4,686,098 to Kopchick et al.; and U.S. Patent No. 4,861,719 to Miller).

Design of retroviral vectors for use in methods of genetic therapy.

Retroviral vectors are presently the preferred vectors for genetic therapy (see, e.g., Anderson (1984) Science 226: 401-409) because retroviral infection is highly efficient and retroviral vectors can be readily modified so that heterologous DNA carried by such vectors is stably integrated into the host cell genome. If retroviral vectors could be produced at a sufficiently high concentration, virtually 100% of exposed target cells, cells that are derived from the afflicted host, could be infected and express integrated proviral and heterologous DNA. Upon infection with a retrovirus, and under appropriate conditions, a single copy of a provirus integrates per cell. Proviral integration is not, per se, harmful to the cell. Also, because of the size and mechanism of retroviral integration, it is possible to know precisely what DNA has been integrated. Finally, retroviral vector systems that have a broad host range are readily available.

Retroviruses consist primarily of a protein envelope that encapsulates core proteins and RNA. The RNA of a retrovirus encodes two long terminal repeat

sequence (LTRs), which include promoter and enhancer regions and which flank the genome; various regulatory signals that regulate transcription, including the CAP site and polyadenylation signals, and that regulate reverse trans-cription and proviral replication; structural genes inclu-ding the env gene, which encodes the envelope proteins, the gag gene, which encodes viral core proteins, and the pol gene, which encodes the reverse transcriptase. The retro-viral RNA also includes signal sequences, such as the tRNA binding site (the replication initiation site for minus DNA strand synthesis), the replication site for plus DNA strand synthesis, and the packaging signal, the psi site.

Retroviral envelope proteins include regions that recognize and specifically bind to mammalian cell surface receptors. Some retroviral envelope proteins only bind to a restricted range of host cells; viruses encapsulated in such envelopes are said to have an ecotropic host range. Other envelope proteins bind to a variety of mammalian cells; viruses encapsulated in such envelopes are said to have an amphotropic host range. Upon specific recognition and binding to host cell receptors, the virus enters the cell. The retroviral reverse transcriptase is translated and the virus is reverse transcribed into a DNA intermediate, referred to as a provirus, which integrates into chromosomal DNA. Proviral DNA can also be replicated and packaged into infectious virions.

Elements for retroviral replication are divided into those that act in cis and those that act in trans. Trans-acting factors include the viral proteins that are necessary for encapsidation, binding and entry of the virus into a target cell, reverse transcription, and

integration of the reverse transcribed DNA into the target cell genome. Cis-acting factors, such as the packaging signal, include those that interact with the trans-acting proteins and other proteins during viral replication (see, e.g., Coffin, J. (1985) in RNA Tumor Viruses, vol. 2, pp. 17-74, R. Weiss et al., eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Some of the cis- and trans-acting functions can be deleted from a retrovirus and, if properly combined, provided separately. A virus that has some or all of the trans-acting functions deleted is replication-incompetent, but, if missing functions are provided, such as by co-transfection with a helper virus containing the necessary functions, packaged defective infectious viral particles can be produced. Alternatively, missing functions can be provided by a cell line, a packaging cell line, that has been modified by stable incorporation of such functions in its genome. Because certain functions can be deleted and provided by way of a helper virus or as part of a packaging cell line, retroviral vectors for delivering heterologous DNA, which is then stably integrated into host cell DNA can be constructed. In addition, by careful design of packaging cell lines and retroviral vectors, it is possible to package infectious replication incompetent retroviral vectors without producing helper virus and thereby provide vectors for integrating DNA into a host cell genome without the concomitant risk of recombinational events between the vector and helper virus that could lead to the production of infectious retroviral particles.

Retroviral vectors are constructed by preparing DNA copies of the retroviral RNA and deleting all or parts of the env, pol and gag genes. Heterologous DNA is

inserted in place of the deleted genes under the control of the endogenous heterologous promoter or other promoter recognized by a host cell RNA polymerase II, or the retroviral 5' LTR. Retroviral vectors, thus, do not include genes for replication. When they are transduced, in the absence of helper virus, into cells that do not include such retroviral sequences, the retroviral vectors cannot replicate and, if properly constructed, they are stably incorporated into the host cell genome. In addition to the LTR sequences and other cis-acting regulatory sequences, retroviral vectors generally also include splice donor and acceptor sites and a selective marker gene, such as the bacterial gene, neo, which encodes neomycin phosphotransferase, which confers resistance to certain antibiotics, under the control of an appropriate eukaryotic promoter.

The host range of the packaged retroviral vector can be controlled by selection of the env gene that is incorporated into the packaging cell line. If an amphotropic host range is desired, then the vector is packaged in a packaging cell line that includes env sequences derived from a retrovirus that has an amphotropic host range. For example, the MoMLV (see, e.g., Mann et al. (1983) Cell 33: 153-159; and Miller et al. (1986) Mol. Cell. Biol. 6: 2895-2902) is an amphotropic retrovirus; its env protein binds to receptors present on most human cells.

Since retroviral vectors do not replicate in the target host cell, retroviral vectors are replicated and packaged in cell lines that include DNA that encodes functions, absent in the vector, that are necessary for packaging and replication. Because of the ease with which retroviruses integrate and excise from chromosomal

DNA and undergo recombination, recombination between the DNA derived from the vector and DNA in the packaging cell line may result in production of packaged, replication competent viruses and/or helper viruses, which encode functions necessary for viral replication. Upon transduction into a target host cell, in the presence of helper viruses, recombination can result in the production of infectious retroviral particles in the host cells. Consequently, for clinical use, not only must retroviral vectors be replication incompetent, the packaging cell line and vector must be designed so that there is virtually no possibility of recombination that could lead to the production of replication competent or helper viruses. This is achieved by carefully designing both the vector and the packaging cell line to include deletions and mutations that would render it highly improbable or impossible for any undesirable recombinational events between the retroviral vector and packaging cell line (see, e.g., Mann et al. (1983) Cell 33: 153-159; Miller et al. (1986) Mol. Cell. Biol. 6: 2895-2902 and (1985) Mol. Cell. Biol. 5: 431; and U.S. Patent No. 4,861,719 to Miller). There is, however, the minute possibility of undesirable recombinational events between the vector and sequences carried on the host cell genome that could result in the production of helper virus or activation of cellular oncogenes. These risks are, however, minute and retroviral vectors have been designed that render the probability of such occurrences insignificant.

Design of packaging cell lines for production of clinically useful recombinant retroviral vectors.

Manipulation of the viral genome has, thus, permitted construction of retrovirus-packaging cell lines

that can produce relatively large amounts of viral vectors in the absence of both replication-competent virus and helper virus. These cell lines package retroviral vector RNA into virions that are capable of infecting a broad range of target host cells, but, that, after infection of such cells, cannot replicate. Packaging cell lines-contain retrovirus-derived DNA that supplies the necessary gene functions, such as the env gene, for viral packaging. Most such packaging cell lines contain helper virus DNA that has been modified by deletion of the packaging signal. It has been found, however, that packaging cell lines in which only the packaging signal is deleted will produce helper virus at low frequency and also interact with some retroviral vectors to yield replication-competent virus at low levels. Thus, additional mutations are introduced into the retroviral DNA in the packaging cell lines in order to further decrease probability for production of helper virus and/or also replication-competent virus (see, e.g., Miller et al. (1986) Mol. Cell. Biol. 6: 2895-2902 and U.S. Patent No. 4,861,719 to Miller).

Packaging cell lines are derived from transformed or immortalized cell lines, such as NIH 3T3 cells, and particularly from NIH 3T3 thymidine kinase (TK^-) cells. A DNA construct, such as a plasmid, containing the retroviral sequences with the desired deletions and mutations and a selective marker, such as the herpes simplex virus (HSV) thymidine kinase (TK) gene are introduced into the cell line, such as the NIH 3T3 TK^- cells, and cultured in selective medium. Cells, which grow in the selective medium, are selected and tested for the presence of the necessary packaging functions. Those that produce retroviral vectors and do not produce helper

viral are selected and used as packaging cell lines to produce infectious replication-incompetent retroviral vectors.

Target cells and their use in genetic therapy.

Suitable target cells for gene transfer are those that readily can be obtained and that persist following transplantation, such as fibroblasts, immune cells, particularly lymphocytes, and epithelial cells (See, e.g, St. Louis et al. (1988) Proc. Natl. Acad. Sci. 85: 3150-54; Keller et al. (1985) Nature 318: 149-154; Miller et al. (1988) J. Virol. 62: 4337-4345; and Morgan et al. (1989) U.S. Patent No. 4,868,116).

The first use of genetic therapy in humans involved tumor infiltrating lymphocytes (TILs) as target cells (see, Rosenberg et al. (1990) New Engl. J. Med. 9: 570-578). TILs are a lymphocyte subpopulation that show promise as vehicles for delivery of anti-cancer therapeutics to tumor sites. These lymphocytes infiltrate into tumors, as part of an attempt by the host's immune system to mount an immunological response.

TIL cells for use as target cells for genetic therapy can be produced in vitro by incubating resected human tumors, such as kidney, colon or breast tumors, melanomas, and sarcomas in vitro in appropriate tissue culture medium that contains interleukin-2 (IL-2). The IL-2 in the medium results in the expansion and activation of T cells within the tumor, the TIL cells, and the destruction of tumor cells or tissue. After 2-3 weeks in culture, the tumor cells have been destroyed and the culture primarily contains lymphoid cells that have the phenotype of cytolytic T lymphocytes (CTL) (see, e.g., Rosenberg et al. (1988) N. Engl. J. Med. 319:1676-1680; Muul et al. (1987) J. Immunol. 138: 989-995; and

Topalian et al., (1989) J. Immunol. 142: 3714-3725).

TIL cells also show promise for use in methods of genetic therapy, particularly cancer therapy, (see, e.g. Culliton (1989), "News and Comment" in Science 244: 1430-1433 and Kasid et al. (1990) Proc. Nat'l. Acad. Sci. 87: 473-477) because they provide a source of autologous cells that target tumors and that can be modified by the insertions of DNA encoding a desired protein, cultured, and reintroduced into the patient. Recently, TILs containing DNA encoding a bacterial marker gene, neo, which encodes neomycin phosphotransferase, were infused into the veins of melanoma patients in order to track the fate of the TILs after infusion in the patients (see Rosenberg et al. (1990) New Engl. J. Med. 9: 570-578). The gene was inserted into a retroviral vector, which was then introduced into a retroviral packaging cell line (Miller et al. (1986) Mol. Cell Biol. 6: 2895-2902). The packaging cell line was cultured and yielded packaged defective virions at titers sufficient to transduce TIL cells at a multiplicity of infection of virions to cells of about 1.3 to 2.3. After transduction, the cells were cultured overnight, and, in an effort to increase the number of cells infected, the TILs were again exposed to the virus. Although only 1 to 11% of the cells were transduced, it was possible to locate and identify the infused TIL in the treated patients for at least 64 days.

TILs from patients with advanced melanoma have been modified by insertion of DNA encoding TNF and will be reinfused into the patients in an effort to enhance the anti-tumor activity of the TIL cells.

The first experiment in genetic therapy for the treatment of a genetic disorder, a severe combined immunodeficiency disease, is presently underway. ADA

deficiency, which is associated with a severe combined immunodeficiency disease, is a fatal condition because the ADA substrates, deoxyadenosine and adenosine, which are toxic to T and B lymphocytes, accumulate in serum of the affected individual. In the hope of treating this disorder, a retroviral vector containing DNA encoding adenosine deaminase (hereinafter ADA) (Hock et al. (1989) Blood 74: 876-881) has been introduced into lymphocytes obtained from a child ADA deficiency.

The retroviral vector was packaged in vitro using a cell line shown to produce relatively high titers of the retrovirus containing the ADA gene without concomitant production of helper virus. Although the titers produced by the packaging cells were relatively high, they were only high enough to infect at a multiplicity of infection of about 1 virion/target lymphocyte, which was sufficient to transduce at most about 10% of exposed target cells after repeated exposures of the target cells to the packaged retroviral vectors. The transduced lymphocytes were then infused into the child. It is hoped that after repeated infusions of similarly transduced lymphocytes that sufficient levels of ADA will be expressed to reduce the concentrations of toxic metabolites and thereby permit development of a normal array of immune cells.

Since lymphocytes have a limited lifespan, infusions of transduced lymphocytes will have to be repeated at regular intervals. For each such infusion lymphocytes will have to be transduced and each transduction will require multiple exposures of the lymphocytes to the packaged retroviral vectors because only relatively low titers of the retroviral vectors can be obtained. Even with as many as six exposures of the

lymphocytes to packaged retroviral vectors, only about 10% of the cultured lymphocytes will be transduced. This procedure is, therefore, costly and, unless improved, will not be available for general clinical use.

Because retroviral particles are fragile, they cannot be concentrated by any means known to those of skill in the art. The concentration (transduced cells/total number of target cells x 100) and total number of transduced target cells that can be obtained is limited by the titer of the retroviral particles produced by the packaging cell line, which in turn is limited by the concentration of packaged particles that are released into the culture medium bathing the packaging cell line. Generally, only titers of from 7 x 10^3 to 5 X 10^5 CFU/ml can be obtained (see, e.g., U.S. Patent No. 4,861,719 to Miller), which severely limits their usefulness in genetic therapy. In order to transduce sufficient numbers of target cells, however, it is necessary to have titers of at least 10^6 to 10^7 CFU/ml) (Miller et al. (1988) J. Virol. 62: 4337-4345)

It is, therefore, an object of this invention to provide a method for producing recombinant viral vectors, particularly recombinant retroviral vectors, at high titers.

It is another object of this invention to provide methods for transducing target cells at a high multiplicity of infection.

It is another object of this invention to provide a dual bioreactor system and methods for efficiently trans-ducing target cells at high multiplicities of infection.

It is another object of this invention to provide high concentrations of transduced target cells

that can be used in methods of genetic therapy.

SUMMARY OF THE INVENTION

Methods for producing of titers of recombinant viral vectors by culturing producer cells in a hollow fiber bioreactor are provided. In particular, methods for producing sustained and continuous production of a high titer of recombinant eukaryotic viral vectors, particularly recombinant retroviral vectors, by culturing a packaging cell line in a hollow fiber bioreactor are provided.

High titers of recombinant retroviral vectors are secreted into the overlying medium, the extra fiber space (hereinafter EFS) medium by producer cells, packaging cells, that are cultured in a hollow fiber bioreactor are also provided.

The ability to produce high titers of recombinant viral vectors permits transduction of sufficient concentrations of target cells to be useful for genetic therapy. Target cells are transduced by contacting the target cells with the EFS medium from the bioreactor in which the producer cells are cultured.

In a preferred embodiment a packaging cell line containg DNA that is derived from a retroviral vector is cultured in the bioreactor. High titers, generally at least about 10^6 CFU/ ml, of infectious packaged retroviral vectors accumulate in the EFS medium. If the EFS medium is harvested and replaced with fresh medium, the producer cells continue to secrete viral vectors at a high rate, generally at least about 10^8 retroviral particles/ml per day. The EFS medium can be repeatedly harvested and production of recombinant retroviral vectors continues and is sustained at the high level.

The EFS medium that contains the recombinant retroviral vectors is contacted with selected target cells, particularly lymphocytes, at a multiplicity of infection that may be as high as about 10 CFU/target cell. The target cells may then be cultured in a second bioreactor. The target cells may be contacted with multiple EFS media harvests from the packaging cell line. It should be possible to transduce up to 100% of the target cells.

In a preferred embodiment, a dual bioreactor system in which the EFS from a first bioreactor in which the producer cells are cultured is connected with the EFS of a second bioreactor in which the target cells are cultured. This assembly efficiently and continuously transduces the target cells by introducing the medium in the EFS from the first bioreactor into the EFS of the second bioreactor. Means for collecting spent EFS in the second bioreactor and adding fresh medium to the EFS of the first bioreactor are included in the system.

Examples are provided that demonstrate that the EFS medium obtained from a hollow fiber culture of a packaging cell line produces high titers of the retroviral vector LASN, which contains nucleic acid that encodes ADA. The packaged LASN retroviral vectors have been used to efficiently transduce ADA-deficient lymphoid cells, which subsequently expressed ADA activity.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1a presents titer (vector particles/ml) as a function of duration of culture in the bioreactor. After 50 days in culture the titer of viral vector particles plateaued at more than 10^6 particles/ml.

Figure 1b presents the same data expressed as packaged retroviral vector particle production per day as

a function of time in culture. Vector particle production per day increases substantially with duration of the culture.

Figure 2 presents a schematic diagram of a dual perfusion circuit for direct and continuous inoculation of the EFS from a hollow fiber bioreactor that contains a viral packaging or producing cell line into the EFS of a hollow fiber bioreactor that contains target cells. The perfusion circuit includes: reservoirs (a), connecting tubing (b), media pumps (c), and hollow fiber bioreactors (d). Cells are injected into the EFS through the loading side-ports (f).

Medium from the EFS of the virus-producing hollow fiber bioreactor is pumped either continuously, periodi-cally or intermittently into the EFS of the target cell hollow fiber bioreactor. The circuit also includes reservoir bottles that contain the EFS replacement medium (1) for the virus-producing bioreactor and for collecting the spent EFS medium (2) from the target cell bioreactor Several smaller reservoir bottles (4) are included in the connecting lines between the EFS of the two bioreactors and between the reservoirs (1) and (2). The smaller bottles may be used for sample withdrawal, inoculation, or for displacement of the EFS medium in either bioreactor with sterile air. There are filters (f) on the each of the reservoirs and smaller bottles. Clamps (X) are present in the various lines to direct the flow of medium. Connectors (C_o), which may be placed in any of the lines, permit removal and replacement of any component of the circuit. An automatic pinch valve, which opens when the peristaltic pump (P) is activated and closes when the pump is not pumping, may also be included in the circuit.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All publications mentioned herein are incorporated by reference thereto. All U.S. patents mentioned herein are incorporated in their entirety by reference thereto.

Definitions

As used herein, genetic therapy involves the transfer of heterologous DNA to the certain cells, target cells, of an individual afflicted with a disorder for which such therapy is sought. The DNA is introduced into the selected target cells in a manner such that the heterologous DNA is expressed and a product encoded thereby is produced. Alternatively, the heterologous DNA may in some manner mediate expression of DNA that encodes the therapeutic product, it may encode a product, such as a peptide or RNA that in some manner mediates, directly or indirectly, expression of a therapeutic product. Genetic therapy may also be used to introduce therapeutic compounds, such as TNF, that are not normally produced in the host or that are not produced in therapeutically effective amounts or at a therapeutically useful time. The heterologous DNA encoding the therapeutic product may be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter the product or expression thereof.

As used herein, heterologous DNA is DNA that encodes RNA and proteins that are not normally produced in vivo by the cell in which it is expressed or that mediates or encodes mediators that alter expression of endogenous DNA by affecting transcription, translation,

or other regulatable biochemical processes. Heterologous DNA may also be referred to as foreign DNA. Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which is expressed is herein encompassed by heterologous DNA. Examples of heterologous DNA include, but are not limited to, DNA that encodes traceable marker proteins, such as a protein that confers drug resistance, DNA that encodes therapeutically effective substances, such as anti-cancer agents, enzymes and hormones, and DNA that encodes other types of proteins, such as antibodies. Antibodies that are encoded by heterologous DNA may be secreted or expressed on the surface of the cell in which the heterologous DNA has been introduced.

As used herein, a therapeutically effective product is a product that is encoded by heterologous DNA that, upon introduction of the DNA into a host, a product is expressed that effectively ameliorates or eliminates the symptoms, manifestations of an inherited or acquired disease or that cures said disease.

Typically, DNA encoding the desired heterologous DNA is cloned into a plasmid vector and introduced by routine methods, such as calcium-phosphate mediated DNA uptake (see, (1981) Somat. Cell. Mol. Genet. 7:603-616) or microinjection, into producer cells, such as packaging cells. After amplification in producer cells, the vectors that contain the heterologous DNA are introduced into selected target cells.

As used herein, operative linkage of heterologous DNA regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences refers to the relationship between such

DNA and such sequences of nucleotides. For example, operative linkage of heterologous DNA to a promoter means that the DNA and the promoter are spatially related such that the transcription of such DNA is initiated from such promoter by an RNA polymerase that specifically recognizes, binds to the promoter and transcribes such DNA. Heterologous DNA may be introduced into a cell by any method known to those of skill in the art.

As used herein, a target cell is a cell into which heterologous DNA is introduced for expression in the host who is being treated. Such heterologous DNA may encode a gene product, such as an enzyme, that certain individuals do not express or express in a form that is defective. Suitable target cells are known to those of skill in the art and include, but are not limited, to fibroblasts (see, e.g., St. Louis et al. (1988) Proc. Natl. Acad. Sci. 85: 3150-54) and immune cells (see, e.g., Keller et al. (1985) Nature 318: 149-154 and Miller et al. (1988) J. Virol. 62: 4337-4345). Target cells may be removed from the individual who is being treated and modified by introducing the heterologous DNA in vitro. For example, target cells, such as lymphocytes may be transduced with retroviral vectors that have been produced by producer cells. Alternatively, it may be possible to modify target cells, such as endothelial cells that line the lungs, in vivo.

As used herein, transduced target cells refers to the portion of target cells that, after contacting target cells with a recombinant vector that includes a heterologous DNA, contain the heterologous DNA or contain the heterologous DNA and express the product of the encoded by the heterologous DNA. Genetically engineered target cells, target cells that contain heterologous DNA,

are used in genetic therapy to correct genetic disorders, such as, but not limited to, certain immunodeficiency diseases, β-thalassemia, Gaucher's disease, hemophilia and cystic fibrosis, by introducing them into an individual who has an inherited or acquired genetic defect. In addition, target cells may be genetically engineered to also express DNA encoding drug resistance, such as methotrexate resistance, or drug sensitivity, such that, when such DNA is expressed, the cells may be selectively expanded or destroyed in vivo and to express thereapeutically effective substances, including antibodies and tumor necrosis factor.

As used herein, transduction is the process whereby a viral vector specifically binds to cell surface receptors and enters the cell. It is a process akin to viral infection, except that viral vectors are modified viruses and, upon introduction, into a target cell, generally, do not cause productive infection. For example, retroviral vectors are generally designed to be replication-incompetent.

As used herein, the concentration of transduced target cells refers to the number of transduced target cells/total number of target cells contacted with the vector. The concentration may be expressed as a percentage (number of transduced target cells/total number of target cells x 100).

As used herein, a recombinant viral vector is a vector that includes DNA that is derived from an RNA or DNA virus and also includes heterologous DNA, which is generally in operative linkage with a promoter and other transcriptional and translational regulatory sequences or signals that are recognized by the host cell in which the virus from which such vector is derived can replicate.

Recombinant vectors may be either retained as part of independently replicating as episomal elements or integrated into the genome of the host cell.

Recombinant viral vectors useful for genetic therapy are typically derived from viruses that infect and replicate in eukaryotic cells and thereby serve as a means for introducing heterologous DNA into eukaryotic cells. Recombinant viral vectors that remain episomal include an origin of replication, whereby DNA synthesis can be initiated. Recombinant vectors that integrate into the genome must include DNA sequences necessary to effect integration. Preferred recombinant viral vectors used for genetic therapy are generally selected from among those which integrate into the host cell genome.

As used herein, a producer cell is cell in which recombinant viruses can replicate and can thereby be amplified. Some producer cells also package and secrete recombinant viruses into the medium in which the cells are cultured. The recombinant viral vectors produced by producer cells are used to transduce target cells. Producer cells are typically immortalized or transformed cell lines that are cultured in vitro and are designed to produce maximal amounts of recombinant vectors. For example, retrovirus packaging cell lines are producer cells that include trans-acting factors necessary to package defective retroviral vectors.

As used herein, adoptive immunotherapy is a therapeutic method, whereby cells of the immune system are removed from an individual, cultured and/or manipulated in vitro, and introduced into the same or a different individual as part of a therapeutic treatment for an acquired or inherited disease. Immune cells and adoptive immunotherapeutic methods may be adapted for use

in methods of genetic therapy as vehicles for delivery of heterologous DNA.

As used herein, immune cells include any cells that participate the functioning of the immune system. Lymphoid cells include lymphocytes, macrophages, and monocytes that are derived from any tissue in which such cells are present. In general lymphoid cells are removed from an individual who is to be treated.

As used herein, a growth promoting substance is a substance, that may be soluble or insoluble, that in some manner participates in, induces cells or otherwise activates cells, directly or indirectly, to differentiate or proliferate. Growth promoting substances include mitogens and cytokines, including interleukins, colony stimulating factors, and any other of such factors that are known to those of skill in the art. For example, many types of cells, including target cells, such as lymphocytes, which in vitro require IL-2, have absolute requirements for certain growth promoting substances. Growth promoting substances are well known to those of skill in the art. Many such substances, such as the interleukins 1-7, have been cloned and expressed in vitro. It is within the level of skill in the the art, to select appropriate growth factors in order for culturing both producer cells and target cells in a bioreactor. Other substances, including polycations, such as protamine, may be added to the bioreactor in order to promote viral infectivity by, for example, enhancing viral adsorption to the target cell surface.

As used herein, a therapeutically effective amount of transduced target cells is a sufficient concentration and number of transduced target cells for at least single infusion of such cells into an individual

for genetic therapy. Upon infusion of a therapeutically effective amount of transduced target cells, a sufficient amount of a product produced by the transduced cells is expressed to ameliorate or eliminate the symptoms or manifestations of an inherited or acquired disease. In order to effect a cure or a substantial reduction of the symptoms or manifestations of the disease, it may be necessary to repeat perform multiple infusions and/or to periodically repeat such infusions.

As used herein, a hollow fiber culture system consists of a hollow fiber bioreactor and means for pumping and collecting perfusing medium. A hollow fiber bioreactor is a hollow shell that encases a plurality of semi-permeable fibers through which medium is perfused.

As used herein, the extra fiber space (EFS) is the space in which the cells grow that is external to the semi-permeable fibers and bounded without by the shell of the hollow fiber bioreactor. The EFS is alternatively referred to as the extra capillary space (ECS).

As used herein, the EFS cell medium is the medium in which the cells in the EFS are growing. It may also be referred to as the EFS supernatant. It contains secreted cellular products, including secreted viral particles, diffusible nutrients and any other compounds, including growth promoting or suppressing substances, such as lymphokines and cytokines, that have been added to the EFS medium, or diffusible products that have been added to the perfusing tissue culture medium. The particular components included in the EFS is a function not only of what is inoculated therein, but also of the characteristics of the selected hollow fiber.

Thus, as used herein, a hollow fiber bioreactor or hollow fiber bioreactor cartridge consists of an outer

shell casing that is suitable for the growth of mammalian cells, a plurality of semi-permeable hollow fibers encased within the shell that are suitable for the growth of mammalian cells on or near them, and the EFS, which contains the cells and the EFS cell supernatant.

As used herein, tissue culture medium includes any culture medium that is suitable for the growth or maintenance of mammalian cells in vitro. Examples of such medium include, but are not limited to AIM-V, RPMI, and Iscove's medium (GIBCO, Grand Island, N.Y.).

As used herein, complete AIM-V is a tissue culture medium that consists of the proprietary formula AIM-V (GIBCO, Grand Island, N.Y.) and also contains 10 μg. gentamicin/ml. (GIBCO), 50 μg. streptomycin/ml. (GIBCO), 50 μg penicillin/ml. (GIBCO), 1.25 μg. fungizone/ml. (Flow Laboratories, MacLean, VA.).

Other suitable tissue culture media are well-known and readily available to those of skill in the art and may be readily substituted for AIM-V.

Hollow fiber bioreactors and hollow fiber culture systems.

Hollow fiber (hereinafter abbreviated as HF) bioreactors and HF cell culture systems are known to those of skill in the art (see, e.g., Knazek et al., U.S. Patent Nos. 4,220,725, 4,206,015, 4,200,689, 3,883,393, and 3,821,087, published international application WO 90/02171, which disclosures are herein incorporated by reference thereto). A HF cell culture system includes the HF bioreactor, pumping means for perfusing medium through the system, reservoir means for providing and collecting medium, and other components, including electronic controlling, recording and sensing devices.

A typical HF cell culture system, such as the CELLMAX™ 100 HF cell culture system (Cellco Advanced Bioreactors, Inc., Kensington, MD.), which is described in PCT International Application WO 90/02171, consists of a standard glass media bottle, which serves as the reservoir, a stainless steel/Ryton gear pump, the autoclavable HF bioreactor, which includes the fibers and shell casing in which cells are cultured, and medical grade silicone rubber tubing, or other connecting means, which serves as a gas exchanger to maintain the appropriate pH and pO_2 of the culture medium. All components are secured to a stainless steel tray of sufficiently small dimensions to enable four such systems to fit within a standard tissue culture incubator chamber. The pump speed and automatic reversal of flow direction are determined by an electronic control unit which is placed outside of the incubator and is connected to the pump motor via a flat ribbon cable which passes through the gasket of the incubator door. The pump motor is magnetically coupled to the pump and is lifted from the system prior to steam autoclaving. Tissue culture medium is drawn from the reservoir, pumped through the lumina of the hollow fibers, and then passed through the gas exchange tubing in which it is re-oxygenated and its pH readjusted prior to returning to the reservoir for subsequent recirculation.

The HF bioreactor, which is a component of a HF cell culture system, is a cartridge that contains a multitude of semi-permeable tube-shaped fibers encased within a hollow shell. The terms HF reactor and HF bioreactor are used interchangeably. HF bioreactors have been used for the growth of mammalian cells and for the production of biologically active products that are

secreted thereby (see, e.g., Knazek et al. supra., see, also, Yoshida et al. U.S. Patent No. 4,391,912) and also have been used to cultivate viruses, including herpes simplex virus, hepatitis virus, equine encephalitis virus, mouse mammary tumor virus and human immunodeficiency virus (see, e.g., Meyers et al. U.S. Patent No. 4,546,083; Markus et al., U.S. Patent No. 4,301,249; Johnson et al. (1978) Appl. Envir. 35:431; and Tsang et al. publication of Poster presentation at Bio-Expo, 1986, Boston MA). HF bioreactors have, not however, heretofore been used for producing recombinant vectors, including recombinant viral vectors, in a form suitable for transducing target cells that are used in genetic therapy.

HF bioreactor cartridges, which are well known to those of skill in the art (see, e.g., PCT International Application WO 90/02171), contain a multitude of tube shaped semi-permeable membranes (hereinafter called fibers) that are encased in a hollow shell. Cultured cells grow and fill the spaces between the fibers and are fed by passage of nutrients through the fiber walls from medium that is perfused through the lumina of the fibers. Any HF bioreactor known to those of skill in the art may be suitable for use to practice this invention. Preferred HF bioreactors for use in accordance with this invention include the B3 and B4 bioreactors (Cellco Advanced Bioreactors, Inc., Kensington, MD) (see, e.g., PCT International Application WO 90/02171 for a complete description of the B3 and B4 bioreactors).

The B3 bioreactor cartridge contains several thousand tube-shaped, semi-permeable membranes, which provide about a 1.1 m^2 surface area. The B4 bioreactor

cartridge is somewhat larger than the B3 cartridge and provides a fiber surface area of about 1.6 m^2. The fibers, which are each approximately 250 μm in diameter, are pulled through a polycarbonate tube that is about 12 inches in length, and the extra-fiber volume is filled at each end with a polymeric material in a manner such that liquid can flow through the lumina of the fibers to exit at the opposite end of the shell. The fiber walls nominally restrict passage to substances having molecular weights less than a desired cut-off range. The selected fiber should be semi-permeable to permit the passage of nutrients into the EFS and should be of a material, such as DEAE-cellulose or polypropylene, on which or in the vicinity of which the mammalian cells are able to grow. For example, the fibers used in the B3 and B4 cartridge are cellulosic hollow fibers whose walls nominally restrict diffusion to substances having molecular weights in the range of 3000 to 4000 Daltons. This molecular weight cut-off range is suitable for use in practicing this invention because it is sufficiently small to prevent diffusion of packaged recombinant viral vectors out of the EFS. The fibers divide the cartridge into the EFS, which is also referred to as the extra-capillary or shell-side space, within which minimal bulk flow of perfusion medium occurs by ultrafiltration through the fiber wall. The EFS volume of the B3 cartridge is about 50 ml and that of the B4 cartridge is about 100 ml.

The particular cartridge selected for use depends upon various parameters, including the requirements of the cells that are being cultivated, the materials perfusing through the lumina of the fibers, and the cellular products and recombinant vectors that are being harvested. It is within the level of skill in the

art to select an appropriate bioreactor cartridge and also HF culture system. The fibers and, therefore, the cartridge and HF cell culture system, are selected as a function of the components of the perfusing medium to which they must be permeable and/or impermeable and as a function of the components of the EFS. In accordance with this invention, the fibers will generally be selected such that they are impermeable to the viral vectors, packaged viral vectors or whatever form the vectors are produced in order to maximize the concentration thereof in the EFS and to prevent undesirable contamination of the perfusing medium with such vectors or virions.

Tissue culture medium perfuses through the lumina of the fibers and is also included within the EFS surrounding said fibers. The tissue culture medium, which may differ in these two compartments, contains diffusible components that are capable of sustaining cell growth and proliferation. Tissue culture medium, which is generally oxygenated, is provided in a reservoir from which it is pumped through the fibers. The flow rate can be controlled by the varying the pump speed. In addition, the direction of flow of the perfusing medium can be reversed (see, e.g., PCT International Application WO 90/02171).

The EFS and/or the perfusing medium may additionally contain an effective amount of at least one growth promoting or suppressing substance, such as IL-2, that specifically promotes the expansion or suppression of the cultured cells, particularly the selected target cells, such as lymphocytes, in which the effective amount is an amount sufficient for the cells to be maintained or proliferate in vitro. The EFS and/or perfusing medium

may also be supplemented with additional ingredients including serum, serum proteins, and selective agents for selecting genetically engineered or modified cells. The selected method is a function of, among other variables, the type of cells, their intended use, and the extent to which they adhere to the fibers.

The flow rate can be increased as the number of cells increases with time. Typically the initial flow rate of the medium is adjusted to about 30 to 40 ml/min and is then increased up to about 300 ml/min as the number of cells increases with time. The direction of perfusion of the medium through the lumina of the hollow fibers may be periodically and automatically reversed, typically every ten minutes, in order to provide a more uniform distribution of nutrient supply, waste dilution, and cells within the space surrounding the hollow fibers.

The entire system is sterilized prior to cell inoculation and is designed for operation in a standard air-CO_2 tissue culture incubator. Upon inoculation, the cells settle onto the surface of the hollow fibers, through which nutrients pass to feed the cells and through which metabolic waste products pass to be diluted into the large volume of the recirculating perfusate. A suspension of cells is inoculated into the extra-fiber space (EFS) of a HF bioreactor usually through one of two side ports. The lumina are perfused with cell culture medium and the cells are maintained <u>in vitro</u> for the desired period of time. As the cells are cultured, the perfusing medium is periodically monitored for glucose concentration. The perfusing medium is replenished by replacing the medium in the reservoir bottles whenever glucose concentration drops to about 30 to 40% of its initial value.

After culturing the cells, the EFS and/or the cells may be harvested. In accordance with this invention, the EFS from a bioreactor that contains producer cells is harvested and used to transduce target cells that are cultured in a second bioreactor. After transduction, which may be repeated multiple times, and incubation, the target cells are harvested. Any suitable means know to those of skill in the art may be used to harvest the EFS and to harvest the target cells. For example, in order to harvest the EFS, the bioreactor cartridge is removed from the incubator and placed in a laminar flow hood. The bioreactor cartridge containing the target cells is given a single gentle shake, which usually suspends about 30-40% of cells, and the contents of the EFS, including the loosened cells, are drained into a side port bottle. Fresh medium is added to the EFS and incubation of the target cells can be continued.

Packaged vector production continues at high rate as long as the EFS is periodically harvested and replaced with fresh medium. Other methods known to those of skill in the art for removing the EFS and cultured cells from the bioreactor may be used.

Selection of the recombinant viral vector and production of high titers thereof.

The preparation and selection of the recombinant viral vector DNA encoding at least one gene product is within the level of skill in the art. In general, the selected recombinant viral vector is one that can be replicated and packaged by selected producer cells but not by the selected target cells. It may be a vector that is integrated into a host cell genome, such as an SV40-derived or retrovirus-derived vector, or one, such

as a vector derived from Epstein Barr virus, which includes an origin of replication, that remains episomal.

The gene product may be a therapeutic product, such as an anti-cancer or anti-viral agent; it may be a product, such as adenosine deaminase or immunoglobulin, that the recipient either fails to produce or produces in a mutated defective form because of a genetic defect; it may be a marker, such as DNA that encodes neomycin or methotrexate resistance, whereby the reinfused target cells may be selected or detected; or it may encode a product that regulates expression of another gene product. Selection, cloning and insertion of the heterologous DNA into the recombinant viral vector is within the level of skill in the art and may be effected by any of the well known methodologies therefor.

Any recombinant viral vector derived from viruses that can replicate in eukaryotic cells may be used. The selected heterologous DNA is inserted into the recombinant viral vectors, which is then introduced into producer cells by any means known to those of the skill in the art. The transfected producer cells are then cultured in a HF bioreactor for a time sufficient for replication and production of the recombinant vector, whereby high titers of recombinant vectors are produced. Preferred embodiments employ recombinant retroviral vectors produced by a packaging cell line that secretes packaged replication-incompetent infectious retroviral particles into the EFS of the HF bioreactor.

Preferred retroviral vectors are those that are suitable for genetic therapy. Suitability for use in genetic therapy necessitates minimizing the possibility for recombination to produce replication competent retrovirus or to activate cellular oncogenes and the

retroviral vector must be packaged in packaging cell lines that do not concomitantly produce helper virus. Such retroviral vectors may be constructed by means known to those of skill in the art may be retroviral vectors known to those of skill in the art or may be derived therefrom.

Typically, suitable retroviral vectors include: the LTRs; necessary regulatory signals and retroviral sequences to produce and integrate proviral DNA into the host cell genome; and heterologous DNA, which includes DNA that encodes a detectable marker and/or selectable marker. The heterologous DNA is inserted in place of all or portions of the retroviral structural genes in operative linkage with transcriptional and translational regulatory sequences including a promoter, such as the 5' LTR or endogenous promoter, that is recognized by an RNA polymerase in the target cell.

Retroviral vectors are generally constructed by preparing cDNA, which is inserted into a convenient plasmid, such as pBR322. Desired insertions and deletions are effected using standard methods, and the plasmids are introduced into selected packaging cell lines in order to generate retroviral particles. Introduction of the plasmids into the packaging cell line may be effected by any method known to those of skill in the art. For example, the DNA may be transfected by Ca-phosphate mediated transfection (see, e.g., PCT International Application WO 90/01870), DEAE-dextran mediated transfection methods, lysozyme fusion, direct uptake or any other method known to those of skill in the art. Typically the plasmids are first introduced into an ecotropic cell line to produce infectious packaged particles, which are then transduced into an amphotropic

packaging cell line, and cultured in selective medium, and cellular clones are selected. The selected clones are tested for the ability to produce packaged retroviral vectors without concomitant helper virus production. Those that do not produce helper virus and/or replication competent packaged retroviral vectors are suitable for use in producing packaged viruses for transducing target cells used in genetic therapy. Examples of retroviral vectors from which clinically useful recombinant retroviral vectors that can be modified by insertion of heterologous include, but are not limited to, the retroviral constructs: pN2 (see, e.g., Keller et al. (1985 Nature 318:149-154; and U.S. Patent No. 4,861,719 to Miller); pLHL, which is derived from N2 (see, e.g., Miller et al. (1986) Cold Spring Harbor Symp. on Quantitative Biology, Volume LI, Cold Spring Harbor Laboratory , pp. 1013-1019); pSDHT (Miller et al. (1986) Somat. Cell. Mol. Genet. 12:175-183), which includes the bacterial marker gene that encodesthe neomycin phosphotransferase gene (neo), which confers resistance to G-418; and pLPL (Proc. Nat'l Acad. Sci. USA 80: 4709-4713), which includes the gene encoding the selective marker hypoxanthine-guanine phosphoribosyltransferase, HGPRT.

Derivatives of these vectors, such as those that include the heterologous gene or genes of interest, may be constructed by inserting selected heterologous DNA into a retroviral vector in operative linkage with a promoter, which recognized by a target cell RNA polymerase, and other transcriptional and translational regulatory signals. For example, retroviral vectors SSC and SSCX, ATCC Accession Nos. 67760 and 67761, respectively, which are derived from N2, encode a soluble

form of the glycoprotein receptor CD4, which as been proposed for use in AIDS therapy. SSC and SSCX have been packaged using the PA317 cell line (ATCC Accession No. CRL 9078). The construct pLPL2 (see, e.g., U.S. Patent No. 4,861,719 to Miller), which is derived from pLPL by including additional deletions, such as deletion of the second packaging signal in the 3′ LTR, which prevents packaging of pBR322 DNA.

In a preferred embodiment, the retroviral vector LASN (see Hock et al. (1989) Blood 74: 876-881), which is a derivative pLNL6, an N2 derivative (see Bender et al. (1987) J. Virol. 61: 1639), and which encodes ADA, is produced in high titer in a HF bioreactor by a packaging cell line derived from PA317 (ATCC Accession No. CRL 9078). The LASN plasmid vector includes DNA that encodes 5′ LTR, the psi$^+$ packaging signal, which includes gag protein encoding sequences, but which have been modified by changing ATG to TAG to prevent translation of any gag encoding sequences (see, e.g., Bender et al. (1987) J. Virol. 61: 1639); the cDNA encoding the ADA gene inoperative linkage with the 5′ LTR; the neo gene in operative linkage with the SV40 early region promoter and enhancers; and the 3′ LTR, which includes a polyadenylation site. The AUG start codon for ADA mRNA begins in the LTR, continues through ADA sequences, the SV40 sequences and neo sequences and terminates in the 3′ LTR. The LASN plasmid vector has been transfected into the packaging line, PA317 (ATCC Accession No. CRL 9078), which produces packaged LASN retroviral particles that have an amphotropic host range (see, Hock et al. (1989) Blood 74: 876-881) but which does not produce detectable helper virus.

In a preferred embodiment, the LASN-producing PA317 cell line is inoculated into the EFS of a HF bioreactor and cultured under conditions whereby packaged LASN retroviral particles accumulate in the EFS medium at high titer. No detectable helper virus is produced.

Preparation of producer cells.

Recombinant viral vectors used for genetic therapy must be able to infect target cells, but should not harm the host. Therefore, the viruses from which they are derived should be modified such that they do not commandeer target cell biochemical pathways to the detriment of the target cell and/or host into which the target cells are introduced. Consequently, in order to amplify recombinant viral vectors that contain heterologous DNA of interest, the recombinant vectors must be cultured in vitro in producer cells in which they can be replicated.

In preferred embodiments, recombinant viral vectors are amplified in producer cells lines, called packaging cell lines, that are cultured in a HF bioreactor and secrete the recombinant vectors into the EFS in a form in which the recombinant vectors can be introduced into target cells. In particular, packaging cell lines that produce infectious, replication-incompetent, recombinant retroviral vectors that contain heterologous DNA are cultured in a HF bioreactor and high titers of packaged retroviral vector particles accumulate in the EFS.

A cell line, such as PA317 that contains such a DNA construct can transmit packaged viral RNAs, including those that encode heterologous DNA, as long as the viral RNA includes the proper cis-acting elements,

such as the packaging signal. The packaged viral particles that are produced by PA317 are amphotropic, and, thus, can infect a broad range of mammalian target cells.

Suitable packaging cell lines may be constructed or may be derived from readily available well-known lines, which include, but are not limited to: psi^2 (Mann et al. (1983) Cell 33 153-159); NIH 3T3 TK$^-$ (Miller et al. (1986) Mol. Cell. Biol. 6: 2895-2902); PA317 (ATCC Accession No. CRL 9078) (see, e.g., U.S. Patent No. 4,861,719); and PE501, which is similar to PA317, but produces packaged retroviral particles that have an ecotropic host range (see, Hock et al. (1989) Blood 74: 876-881).

In a preferred embodiment, the retroviral vector LASN is produced by a packaging cell line that has been derived from PA317. Construction of PA317 is described in U.S. Patent No. 4,861,719 to Miller and construction of the LASN-producing derivative of PA317 is described in Hock et al. (1989) Blood 74: 876-881.

Briefly, DNA constructs, which were carried in pBR322 and in which all cis-acting elements, except for the tRNA binding side, had been deleted were introduced into NIH 3T3 TK$^-$ cells by co-transfection with a selectable marker, the HSV TK gene. TK+ cell clones were selected and tested forthe ability to package and transmit a retroviral vector, pLPL2, that includes the HGPRT gene in operative linkage with a promoter. The retroviral vector was introduced by calcium phosphate-mediated transfection and the cells were cultured. After several days the overlying tissue culture medium was tested assayed for the presence of HGPRT-producing virus. The cell line, PA317, that packaged the highest titer of

HGPRT-virus (6 x 10^4 CFU/ml), was selected.

The retroviral vector, LASN was constructed by introducing cDNA that encodes ADA was inserted in plasmid DNA that contained the retroviral LNL6, which is an N2-derived vector (Bender et al. (1987) J. Virol. 61: 1639). In LASN, ADA-encoding mRNA begins in the 5'LTR, continues through ADA, SV40 and neo sequences and terminates in the 3' LTR (see, e.g., Hock et al. (1989) Blood 74: 876-881) The pBR322-derived plasmid that contained the retroviral constructs was transfected into an ecotropic packaging cell line. Packaged retroviral particles from the ecotropic cell line were transduced into PA317 and G-418-resistant clones were selected and tested for helper virus production. Those that do not produce helper virus have been used to produce packaged LASN for transduction of target cells.

Other retroviral constructs containing heterologous DNA and packaging cell lines may be similarly constructed and used in accordance with this invention.

Culturing producer cells in a HF bioreactor.

Prior to use, a HF cell culture system, such as the CellMAX™ 100, is steam autoclaved, continuously perfused with recirculating deionized water, drained, flushed, and perfused with the selected tissue culture medium in both the EFS and perfusate pathways. All operations are performed in sterile conditions, such as in a sterile laminar flow hood.

A sufficient amount, generally about 10^5 - 2 x 10^6 producer cells/ ml, of cells in a sufficient volume to fill the EFS of a bioreactor cartridge is inoculated into the pre-sterilized cartridge, such as a B3 or B4

bioreactor cartridge (Cellco Advanced Bioreactors, Inc., Kensington, MD),. The inoculated bioreactor is transferred to a standard incubator, perfused with medium at an appropriate temperature, generally about 32° C to about 37° C and maintained under these conditions.

After the cells settle, culture medium is continuously perfused through the HF bioreactor by means of externally applied pressure, such as a pump. A reservoir that contains tissue culture medium, the HF bioreactor cartridge, and pumping means are connected by tubing, typically silicone rubber, which also serves as an oxygenator. The medium may be oxygenated by any means known to those of skill in the art. The silicone rubber tubing simultaneously serves as a membrane gas exchanger to replenish oxygen and, if the medium is buffered with bicarbonate, to maintain the pH via CO_2 transport into the perfusion medium. Medium that is buffered with systems other than bicarbonate do not necessarily require CO_2 in the incubator.

Perfusion is continued for a sufficient time and under conditions, whereby the vector is released into the EFS, which then contains high titers of the recombinant vector. The conditions, which include, the tissue culture medium, incubation temperature and incubation time, are chosen as a function of the requirements of the producer cells and recombinant viral vector. Determination and optimization of such conditions are within the level of skill in the art.

During the incubation period, the reservoir containing the perfusing medium is replaced in order to maintain a sufficiently high concentration of glucose and other diffusible nutrients in the EFS and for waste removal. Typically, the perfusate is replenished several

times a week by replacing the reservoir bottle with one containing fresh medium. Incubation continues for at least about one to thirty days or more days. During the incubation period, the EFS is periodically harvested and contacted with target cells. After the cells have been incubating for one to several days to a week or so, the EFS can be harvested. The EFS can be harvested batchwise, periodically, or continuously or by any variation thereof known to those of skill in the art. It may also be connected to the EFS of a second bioreactor that contains target cells, as shown, for example, in Fig. 2.

In preferred embodiments, LASN-producing PA317 cells, which are suspended in tissue culture medium, are inoculated into a B3 or B4 bioreactor, (Cellco Advanced Bioreactors, Kensington, MD) via the side ports. The bioreactor is attached to a perfusion circuit, the cells are permitted to settle onto the fibers for about 15 minutes to several hours before perfusion is initiated. After perfusing overnight or for day or two the EFS can be harvested, batchwise or by directly introducing it into a second bioreactor using a dual perfusion circuit, fresh EFS medium is added and perfusion continues. If the EFS is harvested periodically, about once a day, the LASN-producing cells continue to produce high titers, at least about 10^5 CFU/ml, for up to at least 6 to 7 weeks.

Viral titer may be measured by any method known to those of skill in the art (see, e.g., U.S. Patent No. 4,861,719 to Miller; and U.S. Patent No. 4,868,116 to Morgan et al. Typically viral titer is measured as colony forming units/ml.

Selecting target cells and transducing them with recombinant viral vectors produced by cells cultured in a HF bioreactor.

Target cells, such as fibroblasts (see, e.g., Palmer et al. (1987) Proc. Natl. Acad. Sci. 84: 1055; St. Louis et al. (1988) Proc. Natl. Acad. Sci. 85: 3150-54 and PCT International Application WO 90/01870) epithelial cells (see, U.S. Patent No. 4,868,116 to Morgan et al.) and immune cells, such as lymphocytes, are obtained either from the patient, who has the inherited or acquired disease or from another donor. The selected target cells are contacted with the harvested recombinant viral vectors that were produced in a HF bioreactor to produce transduced target cells. In order to enhance infectivity of the viral vector, polycations, such as protamine at concentrations of about 5-10 μg/ml, may be added to the harvested viral vectors or to the bioreactor in which the producer cells are cultured. Contacting may be effected by any method known to those of skill in the art. The target cells are then inoculated into the EFS of a second bioreactor, which has been autoclaved and prepared as described above for the producer cells, and incubated as described above.

The target cells may be transduced, either before or after inoculation into the EFS of the second bioreactor, by contacting with the EFS medium from a first bioreactor that contains producer cells. The target cells may be mixed with harvested EFS medium, may be introduced into a second bioreactor into which the harvested EFS medium is inoculated or the EFS of the second bioreactor may be connected to the EFS of a first bioreactor that contains producer cells and is continuously or intermittently inoculated. The target

cells are contacted with harvested vector-containing EFS medium one or more times.

Target cells can be harvested by gently shaking the bioreactor and pouring the suspended cells into a side port bottle. Generally about 10^{10} target cells are used for one treatment and, ideally, 100% should be transduced.

In preferred embodiments, lymphocytes are inoculated into a second bioreactor and then transduced with the EFS medium from a first bioreactor, which contains packaged infectious replication-incompetent retroviral vectors.

In a preferred embodiment, transduction is effected continuously or intermittently using a dual perfusion circuit as shown in Fig. 2, discussed below, by connecting the EFS of the second bioreactor to EFS of a first bioreactor in which retroviral vector-producing cells have been inoculated. The target cells are then repeatedly exposed to EFS medium from the first bioreactor. A high percentage of target cells can thereby be transduced.

The dual bioreactor perfusion circuit.

Continuous or intermittent inoculation of target cells with recombinant retroviral vectors may be effected by directly pumping the EFS from the first bioreactor that contains the producer cells into the EFS of the second bioreactor that has been inoculated with target cells. This may be accomplished using the dual perfusion circuit, pictured in Fig. 2.

As shown in Fig. 2, the perfusion circuits of the two bioreactors are separated by a pinch clamp (represented by ⋈ in Fig. 2). A peristaltic pump, is

used to introduce fresh medium into the bioreactor that contains the producer cells. When the peristaltic pump is operating, the pinch clamp opens, thereby connecting the EFS of the first bioreactor via a side port to that of the second bioreactor via a side port. EFS medium that contains recombinant retroviral vectors is forced out of the first bioreactor through the connecting tubing and pinch clamp and into the second bioreactor. Fresh medium is introduced through the second side port of the producer bioreactor. A filter that is designed to remove blood leukocytes, is placed in the tubing between the two bioreactors, interposed between the pinch clamp and the second bioreactor. This prevents contamination of the target cells by producer cells. Excess medium from the EFS of the second bioreactor is forced through the second side port into an overflow flask. The peristaltic pump may be operated continuously or periodically. In preferred embodiments, the pump is operated for about 1 minute per hour at a pressure sufficient to introduce about 10 ml of vector-containing EFS medium into the EFS of the second bioreactor that contains target cells.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

Production of ADA-containing retroviral particles.

LASN-producing cells were the gift of Dr. Dusty Miller. The construction of the LASN retroviral vector that contains the ADA gene in operative linkage with the LTR and the helper-free cell line that produces packaged LASN retroviral vector particles is described in U.S. Patent No. 4,861,710 to Miller and in Hock et al. (1989) BLood 74: 876-881 is discussed above. The packaging cell

line and LASN vector were prepared using publicly available and well known starting materials.

Briefly, LASN is a derivative of the vector LNL6 (Bender et al. (1987) J. Virol. 61: 1639), which is a derivative of the well-known vector N2 (see, Nature 318: 149-154; see, also, Armentano et al. (1987) J. Virol. 61: 1639). LASN includes, starting at the 5' LTR, which is the MoMLV LTR, the extended packaging signal, psi^+, ADA cDNA under control of the LTR, the SV40 early region promoter and enhancer, the bacterial neo gene under control of the SV40 promoter, the second LTR, and a polyadenylation site. ADA encoding sequences extend through the SV40 and neo and into the 3' LTR. A plasmid containing the LASN sequences was introduced into an ecotropic packaging cell line, PE501, and virus from these cells was used to infect PA317, ATCC accession no. CRL 9078, and G-418-resistant LASN-producing PA317 cells were isolated.

LASN-producing PA317 cells were grown in T-150 flasks and cultured to produce confluent monolayers. The cells from three T-150 culture flasks (about 4.5×10^7 LASN-producing PA317 cells) were trypsinized, resuspended in complete tissue culture medium (cTCM), which contains 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UTAH), and centrifuged at 800 x g for about 10 minutes at room temperature. The cell pellet was resuspended in 100 ml cTCM and inoculated into the EFS of a B4 HF cartridge (Cellco Advanced Bioreactors, Kensington, MD) via the side ports. All operations were performed in a sterile laminar flow hood.

Prior to use, the silicone rubber tubing flow path from the bioreactor culture system had been conn cted to the pump and reservoir and steam autoclaved

with side port tubing and bottles at about 121° C for 20 minutes. Using sterile technique, a B4 bioreactor cartridge was removed from its package and inserted into the sterilized silicone rubber tubing pathway. The side port bottles were attached to the side ports of the B4 HF cell bioreactor. The distilled water in the EFS of the bioreactor was drained into empty side port bottles and discarded. The system was perfused with 0.8 liters of deionized water overnight at 37° C. The perfusion pathway and extra-fiber space of each system were then drained and flushed with Dulbecco's minimal essential tissue culture medium (DMEM, Gibco, Grand Island, NY) which was then discarded and replaced with cTCM, which contained 45 gm glucose/liter DMEM, 10% heat-inactivated FCS, 50 units of penicillin/ml, 50 μg streptomycin/ml, and 2.5 μg amphotericin B/ml, which had been placed in the reservoir of the perfusion circuit. The bioreactor culture system was then transferred to a standard tissue culture incubator, which was held at 37° C and contained a humidified 5% CO_2 in air atmosphere. Perfusion was initiated at a rate of about 100 ml/minute. After an overnight perfusion, the bioreactor was removed from the incubator and the EFS was inoculated with 100 ml of the resuspended LASN-producing PA317 cells via the side port bottles. The entire CELLMAX™ bioreactor unit was then placed into the incubator, but not perfused for 4 hours, in order to facilitate uniform attachment of cells to the fibers. Subsequent perfusion was commenced at a rate of about 40 ml per minute and gradually increased to about 300 ml per minute during the course of the culture in order to insure that the cells were adequately oxygenated. The direction of flow of perfusing medium was not reversed.

Glucose concentration of the perfusing medium was monitored about every 1-4 days. The perfusion medium was replaced when glucose concentration had dropped to about 30-50% of the initial value of about 4.5 gr/l. The medium replacements were performed in a laminar flow hood.

As expected, the perfusate, which was assayed for virus by a colony forming assay, contained no detectable virus. The viral particles are about 150-200 nm in diame-ter, which is too large to diffuse through the fiber walls. At the indicated times (see Figures 1a and 1b), the medium in the EFS, which contained the viral particles, was harvested and replaced with fresh cTCM. The harvested medium was stored at -70 to -80° C awaiting assay for virus content.

Figure 1a presents viral titer as a function of days in culture in the bioreactor. Figure 1b presents the total number of viral particles produced per day as a function of days in culture.

EXAMPLE 2

The effect of harvesting and replacing the EFS on total viral output and viral titer/ml.

On two successive days, day=t and day=t+1, the EFS medium from a bioreactor, which had been inoculated and incubated as described in Example 1, was harvested and titered. The EFS was harvested by gently pressurizing one side port bottle through a 0.2 micron filter to force the contents of the EFS out of the bioreactor and into the other side port bottle.

The first EFS harvest, occurring 22.5 hours after the EFS medium was introduced, yielded a titer of 7.0×10^5 CFU/ml. This was equivalent to a production rate of 5.2×10^7 virus particles per day. After the

harvest in which all of the EFS medium was removed, fresh medium was added to the EFS and incubation was continued for another 4 hours, after which the all of the EFS medium was harvested and titered. This titer was 2×10^5, which is equivalent to production rate of 12.0×10^7 particles per day. All remaining EFS medium was removed and completely replaced by fresh medium. The EFS medium was then harvested after 17 hours and completely replaced with fresh medium, which was harvested after 7 hours. The results are shown in TABLE I.

It was possible to achieve viral titers of 10^5-10^6 and viral production rates from a single bioreactor of more than 10^8 viral particles per day. Furthermore, increasing the frequency of the EFS harvest appeared to increase total viral output.

For comparison, two T-150 flasks containing a pre-confluent monolayer (about 10^7 cells) of LASN-producing PA317 cells were incubated at 37° C in a humidified 5% CO_2 atmosphere. When the cells were confluent, the medium from the monolayer cultures was harvested and titered. The results, which are presented in TABLE II, demonstrated that in monolayer culture, it was only possible to achieve a titer in the range of about 10^3 particles/ml.

TABLE I

PRODUCTION OF VIRAL PARTICLES IN THE HF BIOREACTOR

Consecutive Samples	Duration hours*	titer $x10^{-5}$**	EFS volume	rate of virus production $x\ 10^{-7}$***
1	22.5	7.0	70	5.2
2	4.0	2.0	100	12.0
3	17.0	5.0	75	5.3
4	7.0	8.9	70	21.3

* hours between replacing and harvesting the EFS
** titer= recombinant viral particles/ml
*** rate of recombinant viral particle production = recombinant viral particles/ml/day x EFS medium volume.

TABLE II

PRODUCTION OF VIRAL PARTICLES IN MONOLAYER CULTURE

Flask#/ harvest#	Duration hours*	titer $x10^{-3}$**	volume ml	rate of virus production $x\ 10^{-5}$***
1/1	29	1.67	92	1.27
1/2	27	1	90	.80
2/1	29	1.11	92	.84
2/2	27	.56	90	.44

* hours between feeding and harvesting all of the medium in the flask
** titer= recombinant viral particles/ml
*** rate of recombinant viral particle production = recombinant viral particles/ml/day x volume of supernatant.

EXAMPLE 3

Transduction of lymphocytes with the retroviral particles.

A LASN-producing PA317 culture was initiated by inoculating a B3 bioreactor (Cellco Advanced Bioreactors, Inc. Kensington, MD) with 8.4×10^8 cells, which were 100% viable. The B3 cartridge is similar to the B4 cartridge, except that it smaller. The EFS has a volume of about 50 ml. Tri-lumen, thin-walled tubing was used to connect the cartridge outlet to reservoir in order to increase oxygen transport into the medium. The direction of perfusion of medium was periodically reversed as described in PCT International Application No. WO 90/01271.

The B3 bioreactor was perfused with DMEM that contained 10% heat-inactivated FCS, 2 mM glutamine (Flow Laboratories), 50 units of penicillin/ml and 50 μg streptomycin/ml. The unit was placed in the incubator, as described in Example 1, and the cells were permitted to attach for about 15 minutes. Perfusion was commenced at a low flow rate, less than 100 ml/min. and gradually increased to 300 ml/min. The EFS was harvested periodically as described in Example 2 and centrifuged at about 800 x g for about 10 min. to remove cells and cell debris. The harvested EFS was stored at -70 - -80° C for later analysis.

About five weeks after the culture was initiated, about 60 ml. was harvested from the EFS and replaced with fresh medium. About 10 ml. of the harvested EFS was removed, diluted and checked for bacterial contamination, which was absent, and to check the cells, which appeared healthy.

The remaining 50 ml. was filtered through a 1μ pore size nylon Polydisc™ AS filter (WHATMAN Ltd., Maidstone, England). This filtrate was used for transduction of the target cells.

The selected target cells were a human T cell lymphotropic virus I (HTLV I)-transformed, ADA-deficient, IL-2-dependent, human lymphocyte cell line, TJF-2. TJF-2 was originally obtained from a patient having ADA deficiency and was then transformed with HTLV I.

A second bioreactor, a B4 bioreactor (Cellco Advanced Bioreactors, Inc., Kensington, MD) was inoculated with 1. 3 x 10^8 TJF-2 cells. The cells were cultured in the bioreactor in RPMI 1640 tissue culture medium (Biofluids, Rockville, MD.) supplemented with 10% heat inactivated FCS, penicillin, streptomycin, 2 mM glutamine (Flow Laboratories) and 1000 units/ml of interleukin-2 (IL-2) (provided by Cetus Corp., Emeryville, CA). 1000 units/ml of IL-2 were also included in the EFS. The medium in the reservoir was changed every 1-4 days. Also, more TJF-2 cells were periodically injected into the EFS in order to increase cell density. After the culture was established, 7.2 x 10^8 cells were removed in a laminar flow hood, by giving the bioreactor cartridge a single gentle shake. About 30-40% of cells were then drained into a side port bottle. The cells were about 65% viable. About 20% of the cells were removed to serve as non-transduced controls. The remaining cells were pelleted and resuspended in 45 ml of the LASN filtrate, described above.

The EFS of the second bioreactor, which contained the remaining TJF-2 cells, was reinoculated with the resuspended cells. RPMI 1640 complete m dium,

prepared as described above, was added to fill the EFS, the unit was placed in the incubator and perfusion was started. After 2 days, the transduction procedure was repeated with a second volume of LASN filtrate. After two more days, the transduction procedure was repeated again. Before each transduction with LASN, samples of the cells were removed for analysis. The culture was terminated after about 8 days. It contained a total of 1.02×10^{10} cells with 82% viability.

Some of the sample transduced cells were introduced into soft agar and cultured in the presence of G-418. Colony formation indicated that the cells were expressing the neo gene that is present in the LASN vector.

In addition, samples of TJF-2 cells taken from the second bioreactor before transduction, two days after the first transduction and two days after the second transduction were assayed for the presence of the neo gene by polymerase chain reaction (PCR) analysis, which demonstrated the presence of the gene after transduction.

PCR analysis was carried using the GeneAmp^R Reagents and DNA Thermal Cycler (Perkin Elmer Cetus, Emeryville, CA). DNA was isolated from the transduced and non-transduced TJF-2 cells and PCR was initiated with 1-2 μg of genomic DNA with neo gene primers having the following sequences:

CAAGATGGATTGCACGCAGG

CCCGCTCAGAAGAACTCGTC.

The reaction mixture was heated at 94° C for 2 min., annealed at 56° C for 2 min, and extended at 72 ° C for 3 minutes in the DNA Thermal Cycler for 30 repetitive cycles. The products of the reaction were run on a gel and probed with a neo-specific probe. Genomic

DNA from the transduced cells included neo DNA; whereas the non-transduced cells did not. Therefore, the target cells are transduced by LASN and have LASN-derived DNA incorporated into genomic DNA.

EXAMPLE 4

Transduction of primary, non-transformed, ADA-deficient lymphocytes with LASN using a dual perfusion bioreactor circuit.

A B3 cartridge, prepared as described in Example 1, was inoculated with 3.5×10^8 primary, non-transformed, ADA-deficient lymphocytes, and suspended in 84 ml of the continuously harvested LASN-containing EFS medium. The LASN-containing EFS medium had been harvested during the previous 48 hrs and supplemented with 1000 units of IL-2/ml (Cetus, Emeryville, CA). The bioreactor was perfused overnight with AIM-V containing 1000 U IL-2/ml in the forward direction at a rate of about 100 ml/min.

The EFS of the first bioreactor, which contained the LASN-producing PA317 cells, was then connected to the EFS of the target cell bioreactor in the arrangement of a dual perfusion circuit as shown in Fig. 2. A peristaltic pump pumps fresh medium, DMEM medium containing 10% FCS (HyClone, Logan, UTAH) and 1000 units/ml IL-2, into the entrance side port in the first bioreactor. The perista pump was run for 1 minute every hour. The fresh medium was pumped into the EFS at a rate of 5 mls/min. The LASN-producer PA317 cartridge is perfused with DMEM that contains 2% FCS at a rate of 300 ml/min. As result of the relatively high flow rate of the perfusate and intermittently open side ports, some of the perfusate was ultrafiltered into the EFS through the fiber walls at an approximate rate of about 5 ml/min.

Since serum proteins do not ultrafilter to a significant extent, the final concentration of FCS proteins in the EFS is estimated to be about 5% FCS. When fresh EFS medium is not being pumped into the side ports, the perfusate is not ultrafiltered.

The pinch clamp that separates the EFS of the two bioreactors automatically closes when the pump is off. A RC-50 Pall filter (Pall Biomedical Products Corp., Glen Cove, NY), which is a leukocyte removal filter for blood, is interposed between the first bioreactor and the clamp in order to remove any LASN-producing cells that might be dislodged from the EFS of the bioreactor that contains the LASN-containing EFS medium.

The two bioreactors were sterilely attached using a Sterile Tubing Welder (SCDIIB, DuPont, Wilmington, DE). One side port of the second bioreactor is connected to the input from the EFS of the first bioreactor and the other is connected to a flask, which collects the overflow.

About 3.5×10^8 lymphocytes were inoculated into the second bioreactor and perfused overnight with AIM-V that contained 1000 units/ml of IL-2 at a rate of about 50 ml/min that reversed direction every minute. Because the flow rate of the perfusate in the second bioreactor was relatively slow, there was no substantial ultrafiltration into the overflow flask.

About 19 hours after the second bioreactor was inoculated, the peristaltic pump, which was pumping at a rate of about 5 ml/min, was turned on for one minute and about 10 ml of the EFS medium from the first bioreactor was introduced into the EFS of the second bioreactor. The 10 ml includes 5 ml of fresh medium from the

reservoir and 5 ml resulting from the ultrafiltration of the perfusate into the EFS that occurs by virtue of the relatively high flow rate of the perfusate. Incubation was continued and once an hour the peristaltic pump was automatically turned on for 1 minute. After about 87 hours in culture, including the initial 19 hours, approximately 0.5 x 10^8 lymphocytes from the second bioreactor were harvested by draining them from the EFS.

At the same time, the second bioreactor was inoculated with the lymphocytes, 2 x 10^6 lymphocytes were introduced into each well of two 24 well Costar™ plates. Prior to introduction into the plates, the lymphocytes had been suspended in 1 ml of the continuously harvested LASN-containing EFS medium, which was harvested as described in Examples 1 and 2. Then 1 ml of AIM-V containing 2000 units/ ml of IL-2 was added to each well. The cells were incubated in a CO_2 incubator at 37° C for 87 hours. About 1.6 x 10^8 cells were harvested from the plates at the same time the lymphocytes in the second bioreactor were harvested.

Aliquots of about 10^7 lymphocytes from both the plates and bioreactor were placed in centrifuge tubes, pelleted at 800 x g for 10 min, washed in Hanks BSS, re-pelleted and frozen at -80° C for subsequent ADA protein analyses.

EXAMPLE 5

Measurement of ADA production by the transduced lymphocytes.

Samples of lymphocytes prepared in Example 4 were assayed for ADA production. The cell pellets were warmed and lysed by freeze-thawing. ^{14}C-adenosine, an ADA substrate, was added and the mixture was incubated at 37° C for 1 hr, heat at 95° C for 5 min to quench the

reaction. The mixture is centrifuged and aliquots are spotted onto thin layer chromatography (TLC) paper and run in solvent containing Na-phosphate, saturated ammonium sulfate and N-propyl alcohol for an 1 hr. The TLC paper dried overnight. The spots are cut-out and placed scintillation vials with scintillation fluid and counted in a scintillation counter.

The rate of ADA production by the singly transduced lymphocytes grown in the 24 well plates was 55.2 nmoles/min/10^8 lymphocytes. ADA production by the continuously transduced lymphocyte cells harvested from the second bioreactor was 73.2 nmoles/min/10^8 lymphocytes. This rate of ADA production is comparable to the rate of ADA production, 50-90 nmoles/min/10^8 lymphocytes, by normal lymphocytes that are not deficient in ADA production.

In comparison, the rate of ADA production by lymphocytes transduced using the supernatant obtained from LASN-producing PA317 cells grown in monolayer and exposed to the supernatant six times was only 11.6 nmoles/min/10^8 lymphocytes. The ADA activity of the non-transduced lymphocytes from the ADA-deficient patient was 0.6 nmoles/min/10^8 lymphocytes.

Since modifications will be apparent to those of skill in the art, it is intended that this invention be limited only by the scope of the appended claims.

We claim:

1. A method for producing a high titer of recombinant viral vectors, comprising:

(a) inoculating the extra fiber space (EFS) of a hollow fiber bioreactor with producer cells that release recombinant viral vectors into the EFS medium; and

(b) incubating said producer cells in said bioreactor under conditions whereby the titer of said recombinant viral vectors is sufficiently high to transduce target cells at a multiplicity of infection of more than 1 recombinant viral vector per target cell.

2. The method of claim 1, further comprising harvesting said recombinant viral vectors by collecting the EFS medium.

3. The method of claim 2, further comprising introducing fresh medium into the EFS of said bioreactor and incubating said producer cells under conditions whereby the titer of said viral vectors in said fresh medium is sufficient to infect target cells at a multiplicity of infection of more than 1 recombinant viral vector per target cell.

4. A method for producing a high titer of packaged recombinant viral vectors, comprising:

(a) inoculating the extra fiber space (EFS) of a hollow fiber bioreactor with packaging cell line that releases packaged recombinant viral vectors into the EFS; and

(b) incubating said cells in said bioreactor under conditions whereby the titer of said packaged recombinant viral vectors is sufficient to infect target cells at a multiplicity of infection of more than 1 recombinant viral vector per target cell.

5. The method of claim 4, further comprising harvesting said vectors by collecting said EFS medium.

6. The method of claim 4, further comprising introducing fresh medium into the EFS of said bioreactor and incubating said packaging cells under conditions whereby the titer of said viral vectors in said fresh EFS is sufficient to infect target cells at a multiplicity of infection of more than 1 recombinant viral vector per target cell.

7. The method of claim 6, further comprising repeating said harvesting, introducing, and incubating steps a plurality of times.

8. A method for producing a high titer of recombinant viral vectors, comprising:

(a) inoculating the extra fiber space (EFS) of a hollow fiber bioreactor with a producer cell line that releases recombinant viral vectors into the EFS medium; and

(b) incubating said producer cells in said bioreactor under conditions whereby the titer of said vectors in the EFS medium is at least about 10-fold higher than that produced by said cells when they are cultured in monolayer culture.

9. The method of claim 8, wherein said titer is at least about 10^5 colony forming units/ ml.

10. The method of claim 8, further comprising harvesting said vectors by collecting said EFS medium.

11. The method of claim 10, further comprising introducing fresh medium into the EFS of said bioreactor and incubating said cells under conditions whereby the titer of the recombinant viral vectors in the EFS medium is at least about 10-fold higher than that produced by said cells when they are cultured in monolayer culture.

12. The method of claim 11, further comprising repeating said harvesting, introducing, and incubating steps a plurality of times.

13. A method for producing a high titer of recombinant retroviral vectors, comprising:

(a) inoculating the extra fiber space (EFS) of a hollow fiber bioreactor with a packaging cell line that releases recombinant retroviral vectors into the EFS medium; and

(b) incubating said producer cells in said bioreactor whereby the titer of said vectors is at least about 10-fold higher than that produced by said cells when they are cultured in monolayer culture.

14. The method of claim 13, wherein said titer is at least about 10^5 colony forming units/ ml.

15. The method of claim 7, wherein the rate of production of said packaged vectors is greater than about 10^5 CFU/ml/day.

16. The method of claim 4, wherein said vectors are recombinant retroviral vectors.

17. The method of claim 16, wherein said retroviral vector is LASN.

18. The method of claim 13, wherein said vector is LASN.

19. The method of claim 4, wherein said packaging cell line is derived from PA317.

20. A method of producing a high concentration of transduced target cells, comprising

(a) inoculating the extra fiber space (EFS) of a first hollow fiber bioreactor with producer cells that release recombinant viral vectors into the EFS medium;

(b) incubating said producer cells in said bioreactor for a time sufficient to produce a titer of said recombinant viral vectors in said EFS medium that is sufficiently high to infect target cells at a multiplicity of infection of more than 1 recombinant viral vector per target cell; and

(c) contacting target cells with said EFS medium under conditions whereby said vectors transduce said target cells.

21. The method of claim 20, wherein said vectors are recombinant retroviral vectors.

22. The method of claim 21, wherein said recombinant retroviral vector is LASN.

23. The method of claim 20, wherein said contacting is effected by mixing the EFS medium that contains the recombinant vectors with said target cells.

24. The method of claim 23, wherein said contacting is repeated plurality of times.

25. The method of claim 24, further comprising introducing said target cells into a second hollow fiber bioreactor and incubating said cells under conditions whereby a therapeutically effective amount of transduced target cells are produced.

26. The method of claim 20, wherein said contacting is effected by inoculating the EFS of a second bioreactor that contains target cells with the EFS medium from the first bioreactor that contains the producer cells.

27. The method of claim 26, further comprising incubating said target cells under conditions whereby a therapeutically effective amount of transduced target cells are produced.

28. The method of claim 26, further comprising incubating said target cells under conditions whereby said cells are at least maintained; removing the EFS medium from said second bioreactor; and repeating said inoculating step.

29. The method of claim 26, wherein said removing and inoculating steps are repeated a plurality of times until a therapeutically effective amount of target cells are produced.

30. The method of claim 26, wherein said removing and inoculating steps are repeated a plurality of times until substantially all of said target cells are transduced.

31. The method of claim 26, wherein said EFS of said second bioreactor is inoculated with said EFS medium from the first bioreactor by connecting said bioreactors in a dual perfusion circuit, whereby the EFS of the first bioreactor is connected to the EFS of the second bioreactor.

32. The method of claim 31, wherein said inoculation is effected intermittently.

33. The method of claim 31, wherein said inoculation is effected continuously.

34. The method of claim 20, wherein said producer cells are a retroviral vector packaging cell line.

35. The method of claim 1, wherein said recombinant viral vectors include heterologous DNA that encodes a therapeutically effective product.

36. The method of claim 4, wherein said recombinant viral vectors include heterologous DNA that encodes a therapeutically effective product.

37. The method of claim 20, wherein said recombinant viral vectors include heterologous DNA that encodes a therapeutically effective product.

38. The method of claim 36, wherein said product is selected from the group consisting of adenosine deaminase, tumor necrosis factor, factor VIII, factor IX, interleukin-2, soluble CD4 glycoprotein receptor protein, an antibody, glucose cerebrosidase and the normal product of the gene responsible for cystic fibrosis, Tay Sachs, or Duchenne muscular dystrophy.

39. The method of claim 37, wherein said product is selected from the group consisting of adenosine deaminase, tumor necrosis factor, factor VIII, factor IX, interleukin-2, soluble CD4 glycoprotein receptor protein, an antibody, glucose cerebrosidase and the normal product of the gene responsible for cystic fibrosis, Tay Sachs, or Duchenne muscular dystrophy.

40. The method of claim 20, wherein said packaging cells are derived from PA317.

41. The method of claim 20, wherein said target cells are selected from the group consisting of fibroblasts, immune cells and epithelial cells.

42. The method of claim 41, wherein said cells are lymphocytes.

43. A composition, comprising transduced target cells, wherein said target cells are transduced with recombinant retroviral vectors and the concentration of said transduced target cells is greater than 10%.

44. The composition of claim 43, wherein said target cells are selected from the group consisting of fibroblasts, immune cells, and epithelial cells.

45. The high concentration of transduced target cells produced by the method of claim 20, wherein the

percentage of transduced target cells is greater than 10%.

46. The high concentration of transduced target cells produced by the method of claim 31, wherein the percentage of transduced target cells is greater than 10%.

47. The high concentration of transduced target cells produced by the method of claim 30.

48. A composition, comprising a therapeutically effective concentration of transduced target cells.

49. A dual bioreactor perfusion circuit, comprising:

a first hollow fiber bioreactor and a second hollow fiber bioreactor, wherein the extra fiber space (EFS) of said first bioreactor is connected to the EFS of said second bioreactor by means for intermittently opening said circuit and introducing the EFS medium of said first bioreactor into the EFS of said second bioreactor.

50. The dual bioreactor perfusion circuit of claim 49, further comprising filtering means interposed between said bioreactors for preventing introduction of any cells from the EFS said first bioreactor into the EFS of said second bioreactor.

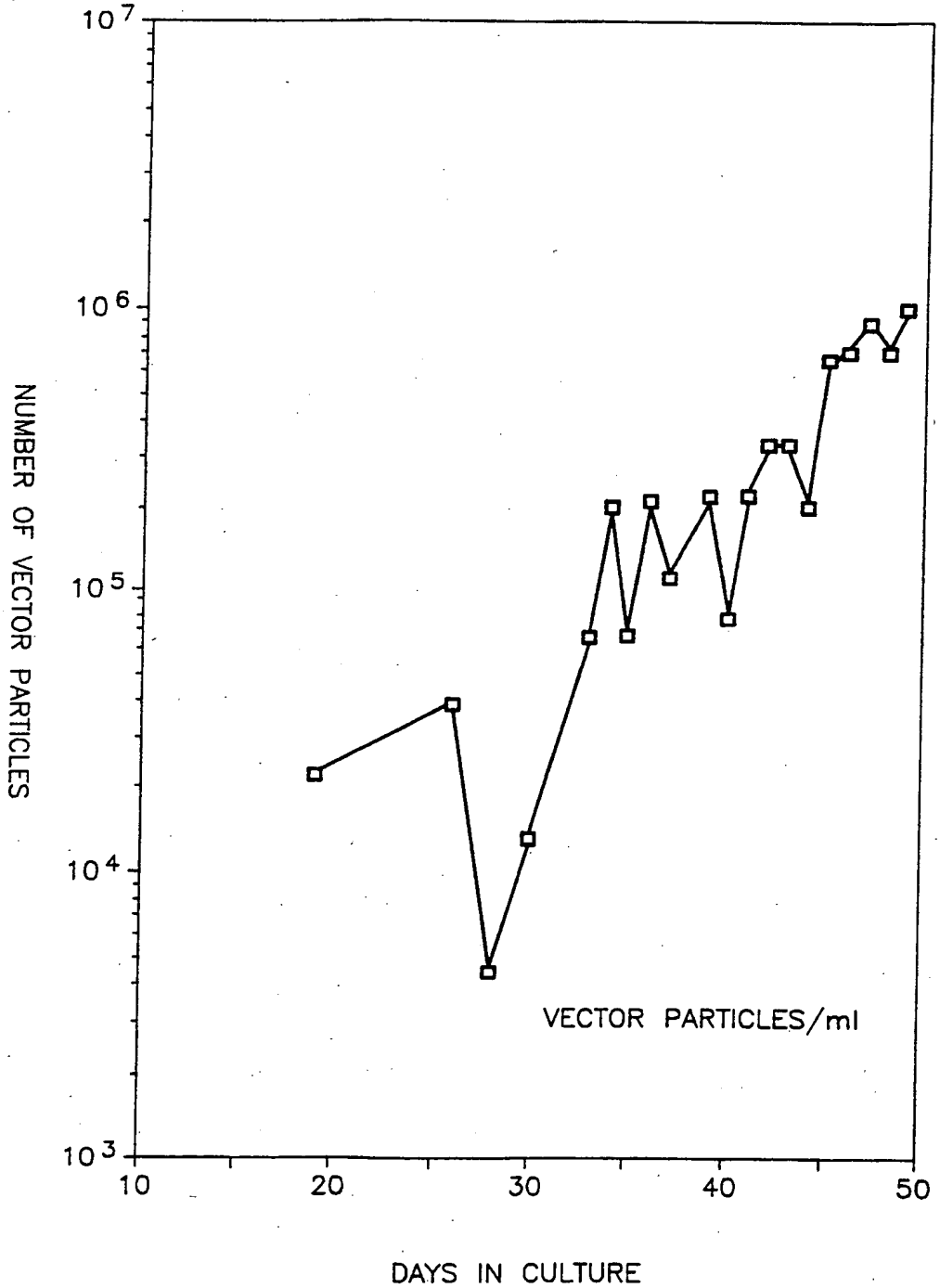
51. A method of producing a high concentration of transduced cells, comprising:

culturing said cells in a hollow fiber bioreactor and continuously introducing a suspension of recombinant viral vectors into the EFS of said bioreactor, whereby a substantial percentage of said cells are transduced by said vectors.

52. The method of claim 51, wherein said recombinant viral vectors are derived from viruses that infect eukaryotic cells.

FIG. 1a

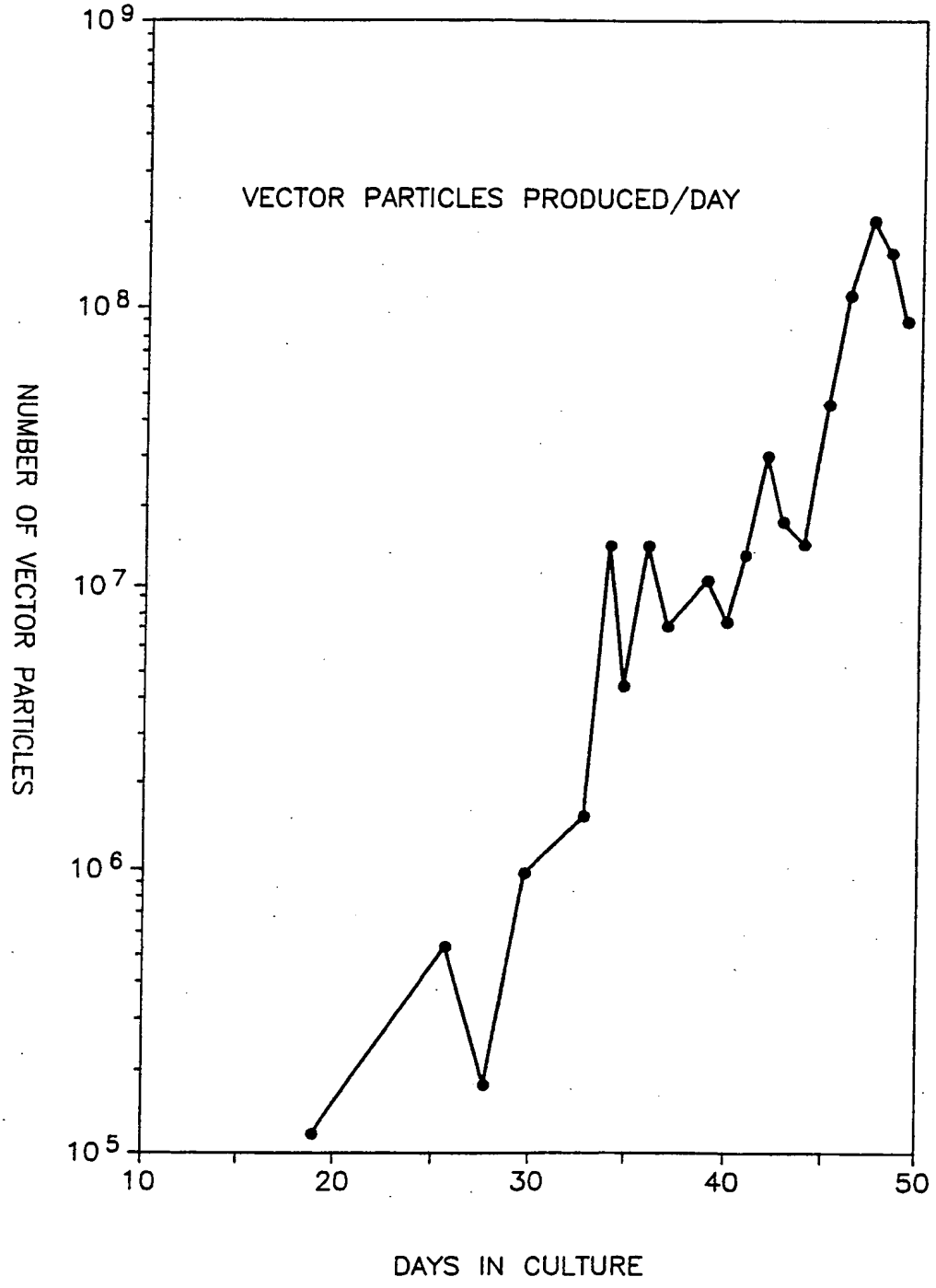
LASN RETROVIRAL VECTOR PRODUCTION



SUBSTITUTE SHEET

FIG. 1b

LASN RETROVIRAL VECTOR PRODUCTION



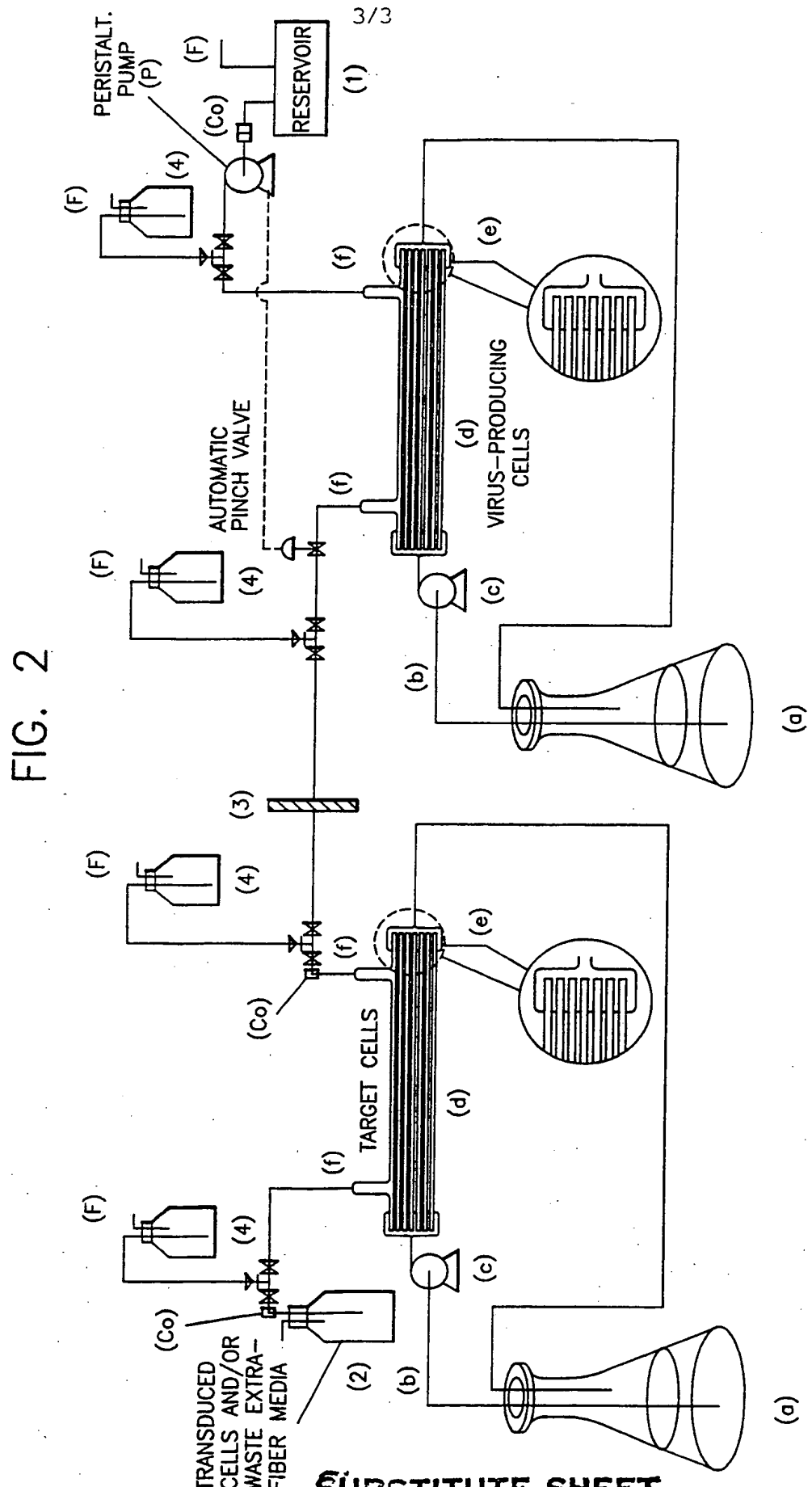


FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/09069

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12N 7/02, 5/00, 5/06; C12M 3/00
US CL : 435/239, 240.1, 240.242, 284

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/239, 240.1, 240.242, 284

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

DIALOG DATABASES: BIOSIS PREVIEWS 1985+, MEDLINE 1975+, NTIS, CA SEARCH, BIOTECHNOLOGY ABSTRACTS 1982+

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US, A, 4,999,298 (Wolfe et al) 12 March 1991. See entire document.	1-52
Y	US, A, 4,861,719 (Miller) 29 August 1989. See entire document.	1-52
Y	US, A, 4,301,249 (Markus et al) 17 November 1981. See entire document.	1-52
Y	Enzyme Microb. Technol., Vol. 9, issued September 1987, M. A. Tyo et al, "Dense cultures of animal cells at the industrial scale," pages 514-520. See entire article.	1-52
Y	Poster presentation at Bio-Expo 86, issued 29 April 1986 (Boston MA), Tsang et al, "Production of HTLV-III Virus in Cells Grown on Artificial Capillaries," No. 510148-1. See entire article.	1-52

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
03 MARCH 1992	17 MAR 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	Johnny F. Railey II

Form PCT/ISA/210 (second sheet)(May 1986) ß

FURTHER INEORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

Please See Attached Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS
(Not for publication)

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-19, 35, 36 and 38, drawn to a first method, for producing a high titer of recombinant viral or retroviral vectors.

II. Claims 20-34, 37, 39-42, 51 and 52, drawn to a second method, for producing transduced target cells.

III. Claims 43 and 44, drawn to a first product, compositions of transduced target cells.

IV. Claims 45-47, drawn to a second product, transduced target cells produced by the methods of claims 20, 30 or 31.

V. Claim 48, drawn to a third product, a therapeutic composition of transduced target cells.

VI. Claims 49 and 50, drawn to a fourth product, a dual bioreactor perfusion circuit.